The Effect of pH, Germinants and Temperature on the Germination of Spores of *Clostridium bifermentans*

By W. M. WAITES AND LINDA R. WYATT

*Agricultural Research Council Food Research Institute, Colney Lane,
Norwich, NOR 70F*

(Received 27 June 1973)

SUMMARY

The germination rate of *Clostridium bifermentans* spores and the manner in which it is influenced by pH, sodium ion concentration and temperature differ according to the organic germinant used. Only L-alanine initiated germination when present alone. The germination rate with optimal L-alanine was increased by L-phenylalanine, L-arginine, L-histidine or L-lysine but not by other common amino acids or L-lactate. With suboptimal concentrations of L-alanine, addition of L-lactate, L-arginine or L-phenylalanine increased the germination rate more than an equal concentration of L-alanine. The mechanisms whereby L-phenylalanine and L-arginine stimulate germination appear to be different from those involved when L-alanine or L-lactate are the germinants.

INTRODUCTION

The optimal conditions for germination of *Bacillus megaterium* spores differ according to the germinant used (Hyatt & Levinson, 1962). These authors studied the influence of pH, temperature and concentration on germination with glucose, glucosamine, L-alanine, L-valine, L-leucine, L-proline or potassium nitrate. No comparable data are available for clostridia. Spores of *Clostridium bifermentans* germinate with sodium ions and either L-alanine or various mixtures of other amino acids (Waites & Wyatt, 1971). This paper reports the effect of conditions on the germination of *C. bifermentans* spores with various germinant systems.

METHODS

Organism, spore preparation and maintenance of culture. The strain of *Clostridium bifermentans* used and the preparation and storage of spores have been described previously (Waites & Wyatt, 1971). The organism was maintained in the reinforced clostridial medium of Hirsch & Grinstead (1954).

Organic germinants. Amino acids were obtained from Koch-Light Ltd, Colnbrook, Buckinghamshire, or from Sigma Biochemicals Ltd, London. L(+) Lactic acid was obtained as the free crystalline acid (grade L-1) from Sigma Biochemicals. Solutions were adjusted to approx. pH 7.5 with KOH and stored at -20°C .

Buffers. Sodium or potassium phosphate buffers were prepared from NaH_2PO_4 and Na_2HPO_4 or KH_2PO_4 and KOH. Quoted pH values were measured by glass electrode after germination was complete.

Spore germination. Germination was followed at 37°C by measuring the decrease in extinction at 600 nm (E_{600}) as described by Waites & Wyatt (1971). As previously, the germination rates given are the maximum rate of decrease in E_{600} (in min^{-1}) expressed as a

Fig. 1. Effect of pH on germination in the 'Ala', 'Ala+' or 'Gly+' system. Spores were incubated at 37 °C, as described in Methods, in the presence of 100 mM-NaCl, plus (mM): 'Ala' (○), i.e. L-alanine (50) and sodium phosphate (83); or 'Ala+' (△), i.e. L-alanine (50), L-arginine (5), L-phenylalanine (5), L-lactate (25) and sodium phosphate (83); or with 'Gly+' (□), i.e. glycine (50) replacing L-alanine in 'Ala+'.

percentage of the initial E_{600} value. Different spore preparations sometimes had different germination rates (for example, the rate with 50 mM-L-alanine varied from 1.5 to 15) but with minor exceptions the relative rates in different germinants were always the same.

Spore dry weight. Dry weights were measured spectrophotometrically using a calibration curve relating E_{600} to spore dry weight.

RESULTS

Effect of pH on germination

With L-alanine alone, over the range covered by sodium phosphate buffer, the most rapid germination occurred at pH 7.8, but addition of L-phenylalanine, L-arginine or L-lactate, or any combinations of these acids, lowered the pH for the most rapid germination to between 7.1 and 7.4 and increased the germination rate by as much as eightfold. Results with L-alanine alone (the 'Ala' system) and with L-alanine + L-phenylalanine + L-arginine + L-lactate (the 'Ala+' system) are shown (Fig. 1). Rapid germination also occurred without L-alanine but with certain mixtures of three or four acids including L-phenylalanine, glycine, L-arginine or L-lactate. With these acids germination occurred most rapidly between pH 6.2 and 6.5 but the germination rate was at least halved by comparison with the 'Ala+' system. The result with L-phenylalanine + glycine + L-arginine + L-lactate (the 'Gly+' system) is also shown (Fig. 1). Subsequent experiments on the effect of sodium ion concentration and temperature were done at these respective pH optima for the three different germination systems.

Fig. 2

Fig. 3

Fig. 2. Effect of sodium ion concentration on germination. Spores were incubated at 37 °C, as described in Methods, in the presence of the concentrations of sodium chloride shown. Concentrations of other germinants are as in Fig. 1 and the pH of potassium phosphate was: with 'Ala' (○), pH 8.0; with 'Ala + ' (△), pH 7.5; with 'Gly + ' (□), pH 6.25.

Fig. 3. Effect of temperature on germination. Spores were incubated at the temperatures shown, in the presence of 'Ala' (○), 'Ala + ' (△) or 'Gly + ' (□) at the same concentrations as in Fig. 1. pH values were as in Fig. 2.

Fig. 4. Effect of different L-alanine, L-phenylalanine, L-arginine and L-lactate concentrations on germination. Spores were incubated at 37 °C, as described in Methods, in the presence of NaCl (100 mM), sodium phosphate (83 mM, pH 8.0) and L-alanine, L-phenylalanine, L-arginine and L-lactate at the concentrations described. Symbols are: L-alanine (○); 150 mM-L-alanine, plus either L-phenylalanine (△) or L-arginine (□); 150 mM-L-alanine plus 5 mM-L-phenylalanine plus L-arginine (▽); and 50 mM-L-alanine, plus L-phenylalanine (▲), L-arginine (■) or L-lactate (●).

Table 1. *Effect of amino and carboxylic acids on germination induced by optimal concentrations of L-alanine*

Spore suspensions were incubated at 37 °C as described in Methods, in the presence of L-alanine (150 mM), NaCl (100 mM), sodium phosphate buffer (83 mM, pH 8.0) and an amino or carboxylic acid (50 mM except where stated). Germination was measured spectrophotometrically and germination rate is calculated from the maximum rate of decrease in E_{600} (in min^{-1}), expressed as a percentage of the initial E_{600} value.

Additional germinant	Germination rate (% min^{-1})
None	32
L-Alanine (to 200 mM final concentration)	33
L-Arginine	40
L-Phenylalanine	37
L-Histidine (40 mM)	37
L-Lysine (40 mM)	36
Pyruvate	33
L-Threonine	33
L-Serine	33
L-Lactate	32
L-Proline	32
L- α -Amino- <i>n</i> -butyrate	31
D-Alanine	30
L-Leucine	30
L-Methionine	24
L-Isoleucine	21

Effect of sodium ion concentration and temperature on germination

With the 'Ala' system no germination occurred in the absence of added NaCl and 160 mM was added for maximal germination, but with the 'Ala+' or 'Gly+' systems rapid germination occurred in the absence of added NaCl and maximal rates were obtained with 25 mM-NaCl (Fig. 2). With the 'Ala' system the most rapid germination occurred at 37 °C but with the 'Ala+' or 'Gly+' system germination was most rapid at 53 °C (Fig. 3).

Effect of germinant concentrations.

No germination was detected in the absence of L-alanine or other germinants and with L-alanine present alone, 50 mM was required to initiate rapid germination (Fig. 4, curve ○—○). The maximal rate of germination with L-alanine present alone occurred at 150 mM. In the presence of 150 mM-L-alanine, the addition of L-phenylalanine, L-arginine, L-histidine or L-lysine but not a number of other amino acids or L-lactate increased the germination rate (Table 1). With 150 mM-L-alanine, 5 mM-L-phenylalanine or L-arginine initiated the maximal rate of germination (Fig. 4, curves $\triangle$ — $\triangle$ and $\square$ — $\square$) and L-arginine still stimulated the germination rate in the presence of 150 mM-L-alanine and 5 mM-L-phenylalanine (Fig. 4, curve ∇ — ∇). With a suboptimal concentration of L-alanine, e.g. 50 mM, addition of L-phenylalanine, L-arginine or L-lactate all increased the germination rate more than the addition of an equal concentration of L-alanine (Fig. 4, solid symbols).

DISCUSSION

The results described here show that the temperature, pH and sodium ion optima for germination of spores of this strain of *Clostridium bifermentans* are dependent on the germinants used. Spores of *C. sporogenes* behave similarly. They germinate optimally at pH 8.5 and 45 °C with L-alanine but at pH 7.5 and 30 °C with L-cysteine (Uehara & Frank,

1965; Tang & Frank, 1972). We have also shown that L-phenylalanine or L-arginine stimulate the germination rate in the presence of optimal or suboptimal concentrations of L-alanine. Other workers have found that additions of amino acids and/or L-lactate increase the germination rate of clostridial spores in the presence of L-alanine (Holland, Barker & Wolf, 1970; Gibbs, 1971; Fujioka & Frank, 1966), while with bacilli, L-phenylalanine and L-proline respectively stimulate the germination rate of *Bacillus cereus* (Wolgamott & Durham, 1971) and *B. megaterium* (Hyatt & Levinson, 1962) in the presence of suboptimal concentrations of L-alanine. However, few studies have compared the effect of an amino acid on germination with both optimal and suboptimal concentrations of another amino acid.

The rapid germination observed with *Clostridium bifermentans* at 53 °C is not unusual, as Wynne, Galyen & Mehl (1955) showed that rapid germination of several clostridia occurred at 75 °C while Levinson & Hyatt (1970) found *Bacillus megaterium* germinated at 50 °C. The requirement for sodium ions for germination has also been demonstrated for aerobic or anaerobic germination of *C. tetani* (Shoesmith & Holland, 1972) and for the germination of another strain of *C. bifermentans* (Gibbs, 1967). More detailed studies have been made with spores of *Bacillus* and, for example, with *B. megaterium* the optimum temperature, pH and germinant concentration for germination depended on the germinant (Hyatt & Levinson, 1962).

In previous work, L-alanine was the only single compound which initiated complete germination of spores of this strain of *Clostridium bifermentans* (Waite & Wyatt, 1971). In the presence of suboptimal concentrations of L-alanine (for example, 50 mM) L-arginine or L-phenylalanine were much more active stimulants than L-alanine. With the optimal concentration of L-alanine (150 mM), L-arginine or L-phenylalanine still stimulated germination and this suggests that they act by a different mechanism to L-alanine. The different pH, sodium ion and temperature optimal for the 'Ala' system, on the one hand, and the 'Ala + ' system, on the other hand, support this suggestion. In contrast, L-lactate stimulated germination in the presence of suboptimal but not optimal concentrations of L-alanine, and this suggests that L-alanine has at least two functions in germination, one of which can be fulfilled by L-lactate. Wax, Freese & Cashel (1967) and Warren (1969) have also suggested that L-alanine may have two functions in the germination of *Bacillus subtilis* and *B. cereus*, and observations on the replacement of L-alanine by fructose (or mannose) + asparagine + glucose for the germination of *B. subtilis* have led Prasad, Diesterhaft & Freese (1972) to suggest that L-alanine may act in three ways, by providing amino groups, NADH and fructose-6-phosphate (or a derivative) to initiate germination. It is possible that in the germination of *C. bifermentans*, L-lactate can give rise to fructose-6-phosphate (via pyruvate) and NADH, while L-alanine acts in the same three ways as in *B. subtilis*.

We are grateful to Dr J. L. Peel for useful discussions and to Miss Jean E. Maskell for technical assistance.

REFERENCES

- FUJIOKA, R. S. & FRANK, H. A. (1966). Nutritional requirements for germination, outgrowth and vegetative growth of putrefactive anaerobe 3679 in a chemically defined medium. *Journal of Bacteriology* **92**, 1515-1520.
- GIBBS, P. A. (1967). The activation of spores of *Clostridium bifermentans*. *Journal of General Microbiology* **46**, 285-291.
- GIBBS, P. A. (1971). Further studies on the germination of spores of *Clostridium bifermentans*. In *Spore Research 1971*, pp. 85-94. Edited by A. N. Barker, G. W. Gould and J. Wolf. London and New York: Academic Press.

- HIRSCH, A. & GRINSTED, E. (1954). Methods for the growth and enumeration of anaerobic spore-formers from cheese with observations on the effect of nisin. *Journal of Dairy Research* **21**, 101-110.
- HOLLAND, D., BARKER, A. N. & WOLF, J. (1970). The effect of carbon dioxide on spore germination in some clostridia. *Journal of Applied Bacteriology* **33**, 274-284.
- HYATT, M. T. & LEVINSON, H. S. (1962). Conditions affecting *Bacillus megaterium* spore germination in glucose or various nitrogenous compounds. *Journal of Bacteriology* **83**, 1231-1237.
- LEVINSON, H. S. & HYATT, M. T. (1970). Effects of temperature on activation, germination and outgrowth of *Bacillus megaterium* spores. *Journal of Bacteriology* **101**, 58-64.
- PRASAD, C., DIESTERHAFT, M. & FREESE, E. (1972). Initiation of spore germination in glycolytic mutants of *Bacillus subtilis*. *Journal of Bacteriology* **110**, 321-328.
- SHOESMITH, J. G. & HOLLAND, K. T. (1972). The germination of spores of *Clostridium tetani*. *Journal of General Microbiology* **70**, 253-261.
- TANG, T. & FRANK, H. A. (1972). Cysteine-induced germination and emergence of putrefactive anaerobe 3679h spores. In *Spores*, vol. 5, pp. 378-383. Edited by H. O. Halvorson, R. Hanson and L. L. Campbell. Washington D.C.: American Society for Microbiology.
- UEHARA, M. & FRANK, H. A. (1965). Factors affecting alanine-induced germination of clostridial spores. In *Spores*, vol. 3, pp. 38-46. Edited by L. L. Campbell and H. O. Halvorson. Ann Arbor, Michigan: American Society for Microbiology.
- WAITES, W. M. & WYATT, L. R. (1971). Germination of spores of *Clostridium bifermentans* by certain amino acids, lactate and pyruvate in the presence of sodium or potassium ions. *Journal of General Microbiology* **67**, 215-222.
- WARREN, S. C. (1969). Spore germination mutants of *Bacillus cereus*. *Journal of General Microbiology* **55**, xviii-xix.
- WAX, R., FREESE, E. & CASHEL, M. (1967). Separation of two functional roles of L-alanine in the initiation of *Bacillus subtilis* spore germination. *Journal of Bacteriology* **94**, 522-529.
- WOLGAMOTT, G. D. & DURHAM, N. N. (1971). Initiation of spore germination in *Bacillus cereus*: a proposed allosteric receptor. *Canadian Journal of Microbiology* **17**, 1043-1048.
- WYNNE, E. S., GALYEN, L. I. & MEHL, D. A. (1955). Thermophilic germination of spores of mesophilic *Clostridium*. *Bacteriological Proceedings*, 39.